WT1 regulates the expression of the major glomerular podocyte membrane protein Podocalyxin

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The *WT1* tumor suppressor gene encodes a zinc finger transcription factor expressed in differentiating glomerular podocytes. Complete inactivation of *WT1* in the mouse leads to failure of mesenchymal induction and renal agenesis, an early developmental phenotype that prevents analysis of subsequent stages in glomerular differentiation [1]. In humans with Denys-Drash Syndrome, a heterozygous germline mutation in *WT1* is associated with specific defects in glomeruli and an increased risk for developing Wilms Tumor [2, 3]. WT1 target genes implicated in cell cycle regulation and cellular proliferation have been proposed [4], but the link between WT1 function and glomerular differentiation is unexplained. Here, we show that inducible expression of WT1 in rat embryonic kidney cell precursors leads to the induction of endogenous Podocalyxin, the major structural membrane protein of glomerular podocytes, which is implicated in the maintenance of filtration slits. Binding of WT1 to conserved elements within the *Podocalyxin* gene promoter results in potent transcriptional activation, and the specific expression pattern of Podocalyxin in the developing kidney mirrors that of WT1 itself. These observations support a role for WT1 in the specific activation of a glomerular differentiation program in renal precursors and provide a molecular basis for the glomerulonephropathy that is characteristic of Denys-Drash Syndrome.

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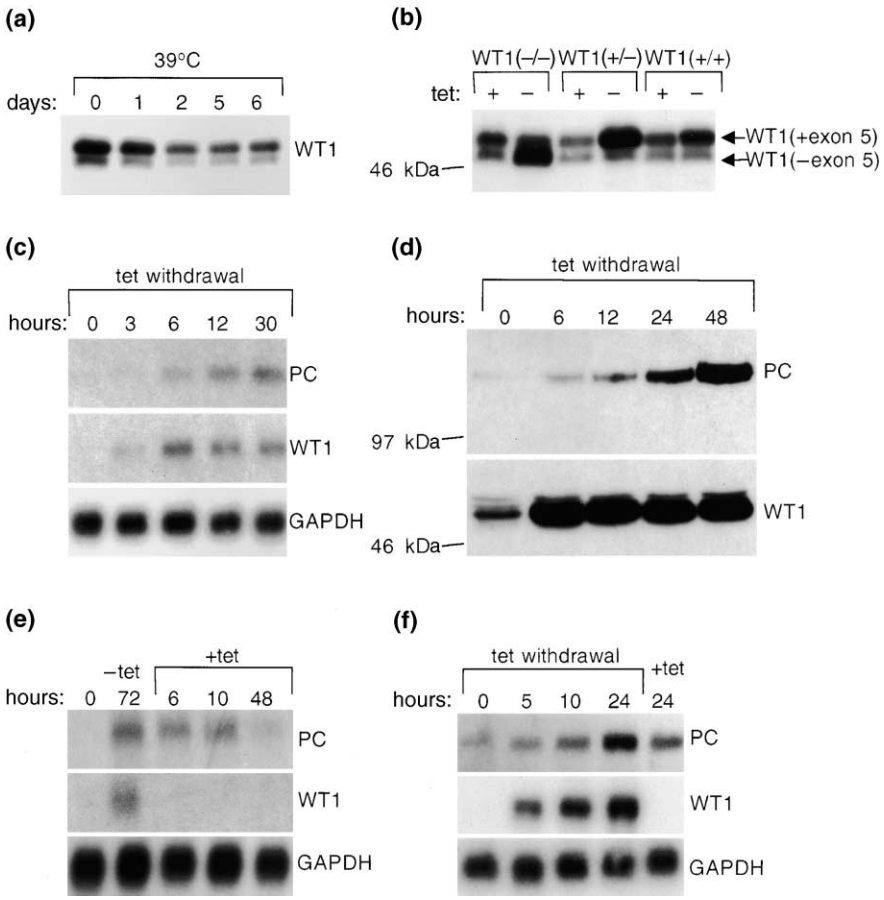
Results and discussion

To establish an appropriate cell type in which to identify physiological targets genes of WT1, we made use of RSTEM cells, rat embryonic kidney cells dissected from the undifferentiated renal mesenchyme at embryonic day 12.5, preceding invasion by the ureteric bud and induction of differentiation [5]. These cells were reversibly transformed using temperature-sensitive SV40 large T antigen, allowing long-term in vitro culture at the permissive temperature (32°C). Inactivation of T antigen by a temperature shift to 39°C restores their differentiation potential, demonstrated by their ability to contribute to the formation of mature kidney structures after reinoculation into 12.5-day kidney rudiments [5]. Consistent with their metanephric mesenchymal origin, RSTEM cells express endogenous WT1 when cultured at 32°C, with a progressive reduction in expression following a temperature shift to 39°C (Figure 1a).

We generated multiple independent RSTEM cell lines in which stable expression of a tetracycline-repressible promoter allows tightly regulated inducible expression of the different WT1 isoforms. WT1 is encoded by distinct alternatively spliced transcripts, of which the most significant is the variable insertion of three amino acids, lysine, threonine, and serine, or "KTS", between zinc fingers three and four [6]. WT1(−KTS) binds to a defined DNA consensus sequence and mediates transactivation of responsive promoters, while WT1(+KTS) fails to bind this sequence and has been implicated in some aspect of RNA processing [7]. The presence or absence of 17 amino acids encoded by *WT1* exon 5 results from a separate alternative splicing event that allows electrophoretic distinction between the WT1 isoforms. RSTEM cells with inducible expression of WT1 (+/−KTS) (+/− exon 5) were generated, with ectopic protein expression on the average of 5-fold over the baseline at 32°C and 20-fold over the baseline at 39°C (Figure 1b,d). Induction of none of the WT1 isoforms was associated with gross phenotypic changes or any immediate effects on cellular proliferation, facilitating the search for direct transcriptional targets.

We first tested a number of previously reported candidate target genes to determine whether their expression was modulated by inducible WT1(−KTS). We confirmed the induction of Amphiregulin by WT1(−KTS) in RSTEM cells [8]. By inducing one isoform (+/− exon 5) of WT1(−KTS) and then examining the electrophoretically distinct endogenous isoform (+/– exon 5), we did not

Figure 1



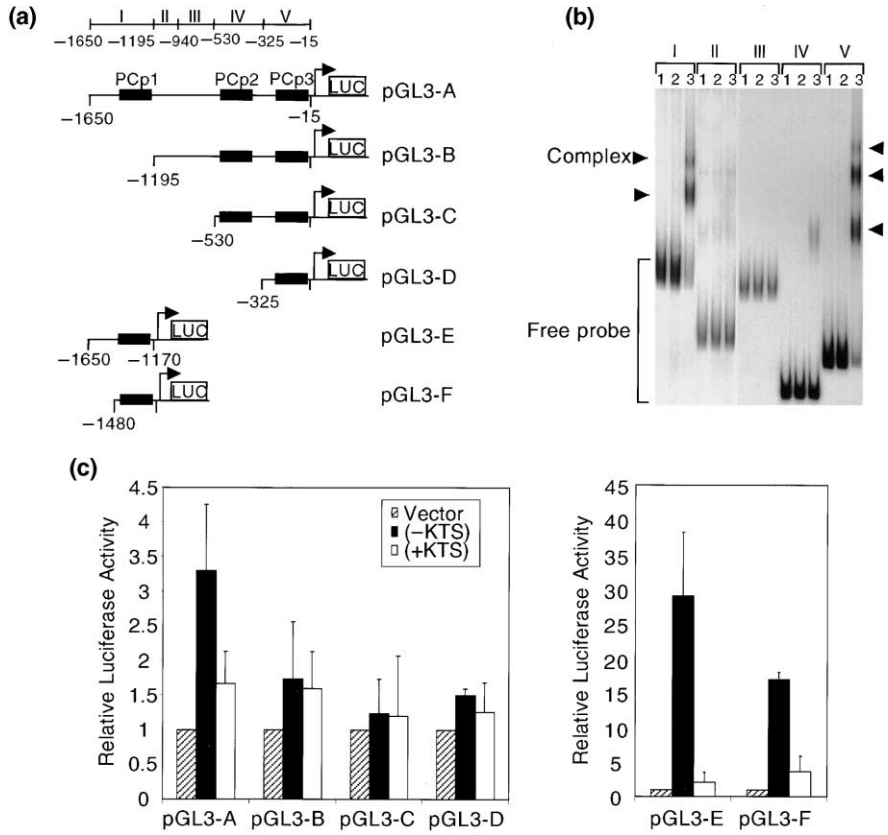
Inducible expression of WT1 splice variants in RSTEM cells. **(a)** Expression of endogenous WT1. Western blot analysis of RSTEM cells cultured at 32°C and following a temperature shift to 39°C. **(b)** The absence of endogenous WT1 autoregulation following ectopic tetracycline-regulated expression of WT1 isoforms. Western blot analysis of RSTEM cells cultured at 32°C in tetracycline (tet +; no expression) or 18 hr following tetracycline withdrawal (tet −) to induce distinct WT1 isoforms: (−/−), lacking exon 5 and KTS splice insertions; (+/−), encoding exon 5 but lacking KTS; (+/+), encoding both exon 5 and KTS. **(c)** Induction of PC mRNA by WT1(−KTS) in RSTEM cells. Northern blot analysis of RSTEM cells (39°C) with regulated expression of WT1(−KTS) at sequential times following the withdrawal of tetracycline. Blots were hybridized for PC, WT1, and GAPDH (loading control). **(d)** Induction of PC protein by WT1(−KTS). Western blot analysis of RSTEM cells (39°C) at sequential intervals following tetracycline withdrawal and WT1(−KTS) induction. **(e)** Reversibility of PC induction in RSTEM cells. Northern blot analysis of cells at sequential intervals following the readdition of tetracycline and the suppression of ectopic WT1(−KTS) expression. **(f)** Induction of PC by WT1(−KTS) in heterologous cell type. Northern blot analysis of U2OS osteosarcoma cells with tetracycline-regulated WT1(−KTS) after the withdrawal of tetracycline for the indicated times. In the last lane, tetracycline was removed for 24 hr and then added back for another 24 hr.

confirm the previously postulated autoregulation of WT1 expression (Figure 1b) [9]. We were also unable to confirm changes in the expression of a number of other previously proposed WT1 targets, including *EGR1*, *IGF2*, *PDGF-A*, *Bcl2*, *IGFR*, *c-myc*, and *EGFR* (data not shown; [4]). In addition, we tested for the WT1 regulation of genes that play important roles in early kidney development, some of which have been postulated to interact with WT1, including the transcription factors Pax2 and Pax8 and the secreted factors BMP7, GDNF, and Wnt4. Endogenous expression of these genes was not altered following the induction of WT1(−KTS) in these kidney precursor-derived cells (data not shown), suggesting that their physiological expression in the renal mesenchyme is unlikely to be directly regulated by WT1.

During kidney development, WT1 expression peaks within glomerular podocyte precursors [10]. This, along with the glomerular defects found in Denys-Drash (DDS) patients, prompted us to examine podocyte markers in RSTEM cells following inducible expression of WT1. The best-characterized marker is Podocalyxin (PC), a highly specialized transmembrane sialoprotein that is thought to contribute to the formation of slit diaphragms via the separation of podocyte "foot processes", thereby allowing filtration of blood through the glomerulus [11, 12]. PC expression was measured by Northern and Western blot analysis. Rapid and dramatic upregulation of PC was observed following the induction of WT1(−KTS), with downregulation of expression upon the readdition of tetracycline (Figure 1c–e). Inducible expression of the transcriptionally inactive WT1(+KTS) isoform had no effect on PC expression (data not shown). In contrast to PC, other genes expressed in podocytes, including *Podoplanin*, *5-1-6*, *Synaptopodin*, *Nephrin*, *Podocin*, α*-actinin-4*, *CD2AP*, and *Desmin*, were not induced by WT1 (data not shown). The induction of endogenous PC following inducible expression of WT1 was not restricted to RSTEM cells but was also observed in heterologous cell types, including the U2OS osteosarcoma cell line (Figure 1f). In both RSTEM and U2OS cells, the rapid and reversible induction of PC by WT1(−KTS) suggests a direct transcriptional mechanism, rather than an indirect consequence of a complex cellular differentiation pathway.

To study the regulatory sequences present within the *PC*

Figure 2

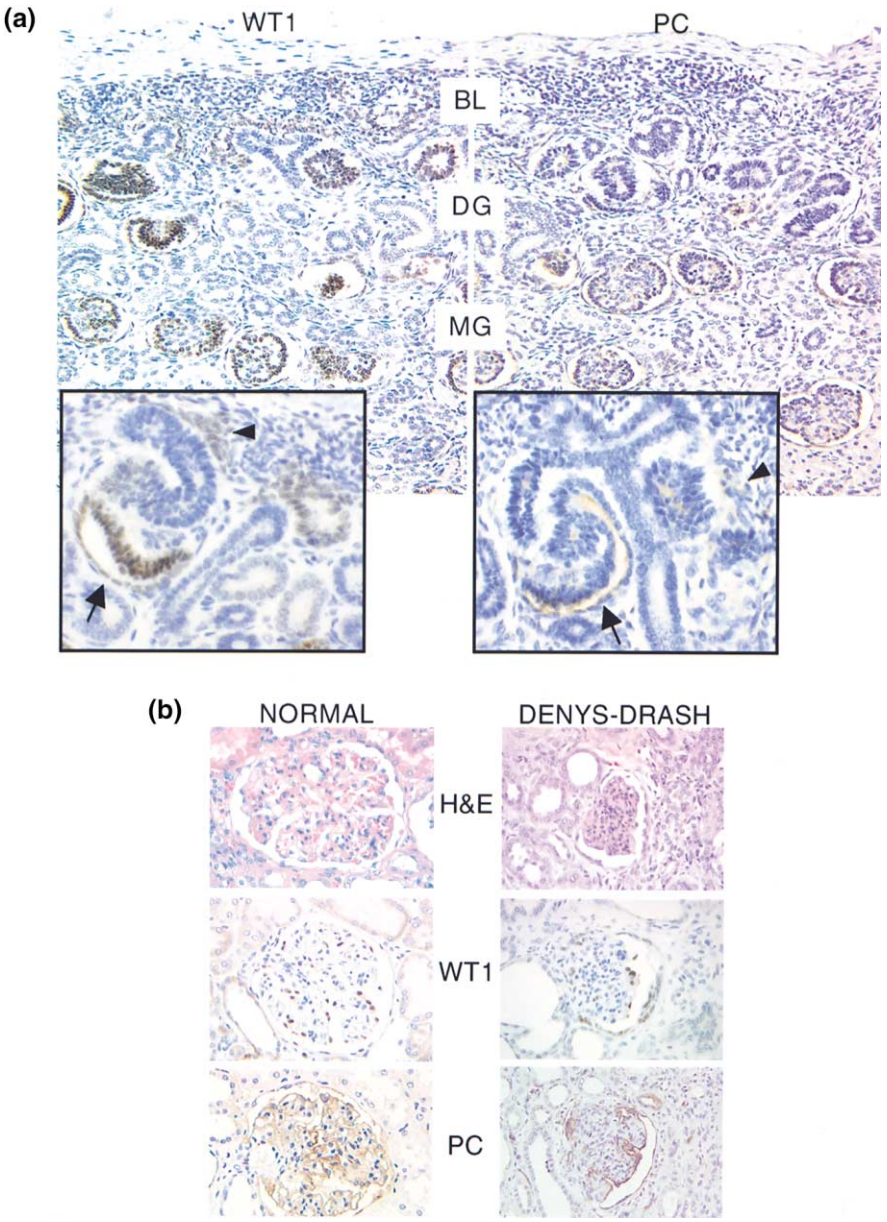


Identification of a WT1-responsive element within the *PC* promoter. **(a)** A schematic representation of the *PC* promoter (GenBank accession number AF395890), denoting the positions of potential WT1 binding sites: the nested WTE sites PCp1 (−1213 to −1227), the single WTE site PCp2 (−495 to −504), and the GC-rich sequence PCp3 (−57 to −83), indicated by black boxes. All reporter constructs shown (A–F) were generated in the promoterless luciferase vector, pGL3. Fragments I–V indicate regions used for EMSA. **(b)** EMSA analysis of the five *PC* promoter fragments (I–V), following incubation with the zinc finger domain of either WT1(+KTS) (lane 2), WT1(−KTS) (lane 3), or probe alone (lane 1). End-labeled probes were incubated with 500 ng of the respective GST fusion proteins. Migration positions are shown for unbound probes of various sizes (bracket); multiple shifted bands seen with probes I and V (arrowheads) may represent the ability of >1 molecule of WT1 to bind to each fragment. **(c)** Activation of the *PC* promoter by WT1(−KTS). Luciferase activity, relative to vector-transfected cells, was measured in NIH3T3 cells following cotransfection of 1 μg WT1 (− or +KTS) and the indicated *PC* reporter constructs (0.2 μg). Standard deviations were derived from three independent experiments. Results were standardized for transfection efficiency using a cotransfected reporter (Renilla luciferase). Equal amounts of DNA were present in each transfection.

gene promoter, we identified a human bacterial artificial chromosome (BAC) spanning the 5′ untranslated region of the transcript. Insertion of 1.6 kb of sequence upstream of the transcriptional start site of *PC* into the promoterless luciferase reporter plasmid, pGL3, consistently led to 3-fold transcriptional activation following transient transfection of WT1(−KTS) into NIH3T3 cells, a recipient cell line that does not express endogenous WT1 (Figure 2c). The transcriptionally inactive WT1(+KTS) had no effect. We used a combination of nested deletion constructs and electrophoretic mobility shift assays (EMSA) to map the WT1(−KTS)-responsive domain (Figure 2a). Deletion of nucleotides −1196 to –1650 abrogated WT1-mediated transcriptional activation of the reporter (Figure 2c). This sequence contains a potential binding site, PCp1 (nucleotides –1213 to −1227: 5′-GGTGGGAGTGGG TGT-3′ in the antisense strand), which contains two overlapping WTE elements, high-affinity WT1(−KTS) binding sites that were identified by PCR-selection experiments and are present in the WT1-responsive *Amphiregulin* promoter [8, 13]. Transfection of a minimal reporter containing a single copy of PCp1 resulted in 20- to 30-fold transcriptional activation following the coexpression of WT1(−KTS) (Figure 2c). We identified two other potential binding sites in the *PC* promoter: PCp2 (nucleotides −495 to –504: 5′-AGGTGGGAGA-3′ in the sense strand), containing a single WTE element, and PCp3 (nucleotides −57 to –83: 5′-GCGGGGGCGGGGGCGG GGGCGGGGGCC-3′ in the sense strand), containing four overlapping EGR1 sites, which comprise a GC-rich motif also recognized by WT1(−KTS) [14]. However, while EMSA analysis demonstrated binding of bacterially synthesized WT1 zinc fingers to all three potential binding sites (Figure 2b), deletion of either PCp2 or PCp3 had a minimal effect on transactivation of the *PC* promoter reporter, and neither sequence demonstrated WT1(−KTS)-mediated transactivation when introduced into a minimal reporter construct (Figure 2c and data not shown). Taken together, these experiments suggest that the two overlapping WTE elements that constitute PCp1 are primarily responsible for the activation of the *PC* promoter by WT1(−KTS).

Both WT1 and PC have specific expression patterns within the developing nephron [10, 15]. To compare their developmental time course of expression, we analyzed adjacent sections of fetal kidney representing the successive stages of renal differentiation from the initial condensing subcortical mesenchyme to the mature tubules and glomeruli (Figure 3a). Consistent with previous obser-

Figure 3



Colocalization of WT1 and PC in the developing kidney. **(a)** Immunohistochemical analysis of adjacent sections from 20-week human kidneys stained with antibodies against WT1 or PC. Both antibodies faintly stain early mesenchymal cells within the blastemal zone (BL). High-level expression of both WT1 and PC is evident in developing glomeruli (DG) and mature glomerular (MG) podocytes (×200). Inset: higher magnification (×400) to illustrate WT1 and PC expression in undifferentiated blastemal cells (arrowhead) and in podocyte precursors of an S-shaped body (arrow). **(b)** High magnification (×400) of glomeruli from normal and DDS kidneys from a 3-year-old child stained using hemotoxin and eosin (H&E), anti-WT1, or anti-PC antibodies. The few glomeruli that do form in DDS patients are sclerotic and have decreased staining for both WT1 and PC.

vations, low levels of nuclear WT1 are first detectable by immunohistochemistry within the induced and uninduced metanephric mesenchyme. As patterning of the nephron proceeds, WT1 expression peaks and becomes restricted to the presumptive glomerular visceral epithelial cells of developing glomeruli. WT1 expression is maintained as these cells develop into mature podocytes. Expression of PC follows that of WT1, with expression first evident in the condensed blastema and podocyte precursors. Expression levels peak and are maintained in the developing and mature glomerular podocytes. The superimposition of temporal and spatial WT1 and PC expression patterns supports a physiological interaction. In addition to colocalization within specific structures of the developing kidney, PC and WT1 are also coexpressed in mesothelium and hematopoietic precursors [10, 16–18].

The renal parenchyma of patients with DDS shows extensive glomerulosclerosis, nephritis, and effacement and fusion of podocyte foot processes [3]. Immunohistochemistry demonstrates reduced numbers of glomerular cells with reactivity for WT1 and PC (Figure 3b). The possibility that a reduction in PC expression accompanying the constitutional WT1 defect in DDS may contribute to the glomerular abnormalities that define this syndrome is supported by a number of observations. In vivo treatments that disrupt the negatively charged extracellular domain of PC lead to the effacement of foot processes and renal

failure [19], and ectopic expression of PC in cultured kidney cells suppresses intercellular aggregation, consistent with an antiadhesion role for this protein [20]. While this manuscript was under review, Doyonnas et al. reported the inactivation of *PC* in mice by homologous recombination [16]. *Pc* null mice die within 24 hr of birth of anuric renal failure attributed to the persistent intercellular association of podocytes via tight junctions and their failure to form foot processes and slit diaphragms.

The identification of *PC* as a physiological target gene of WT1 points to a direct role for WT1 in the activation of a terminal differentiation program in glomerular podocytes, a stage of kidney development that is not attained in *wt1* null mice. The apparent role of WT1 in differentiating glomeruli is consistent with the recent observation that WT1 directly induces lineage-specific hematopoietic differentiation [18]. While these properties are most clearly defined for the transcriptionally active WT1(−KTS) isoform, the contribution of WT1(+KTS) remains poorly understood. Patients with Frasier syndrome and recently generated mouse models, both of which display selective reductions in WT1(+KTS) expression, also demonstrate glomerular abnormalities [21, 22]. Identifying downstream effectors of WT1(+KTS) will be essential to understanding its contribution to kidney differentiation.

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